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Biogeography, Host Specificity, and Molecular Phylogeny of the Basidiomycetous Yeast *Phaffia rhodozyma* and Its Sexual Form, *Xanthophyllomyces dendrorhous*▽

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Phaffia rhodozyma **(sexual form, *Xanthophyllomyces dendrorhous*) is a basidiomycetous yeast that has been found in tree exudates in the Northern Hemisphere at high altitudes and latitudes. This yeast produces astaxanthin, a carotenoid pigment with biotechnological importance because it is used in aquaculture for fish pigmentation. We isolated *X. dendrorhous* from the Southern Hemisphere (Patagonia, Argentina), where it was associated with fruiting bodies of *Cyttaria hariotii*, an ascomycetous parasite of *Nothofagus* trees. We compared internal transcribed spacer (ITS)-based phylogenies of *P. rhodozyma* and its tree host (Betulaceae, Corneaceae, Fagaceae, and Nothofagaceae) and found them to be generally concordant, suggesting that different yeast lineages colonize different trees and providing an explanation for the phylogenetic distance observed between the type strains of *P. rhodozyma* and *X. dendrorhous*. We hypothesize that the association of *Xanthophyllomyces* with *Cyttaria* derives from a previous association of the yeast with *Nothofagus*, and the sister relationship between Nothofagaceae and Betulaceae plus Fagaceae correlates with the phylogeny of *X. dendrorhous* strains originating from these three plant families. The two most basal strains of *X. dendrorhous* are those isolated from *Cornus*, an ancestral genus in the phylogenetic analysis of the host trees. Thus, we question previous conclusions that *P. rhodozyma* and *X. dendrorhous* represent different species since the polymorphisms detected in the ITS and intergenic spacer sequences can be attributed to intraspecific variation associated with host specificity. Our study provides a deeper understanding of *Phaffia* biogeography, ecology, and molecular phylogeny. Such knowledge is essential for the comprehension of many aspects of the biology of this organism and will facilitate the study of astaxanthin production within an evolutionary and ecological framework.**

Phaffia rhodozyma was originally isolated in the late 1960s, by Phaff and collaborators, who collected 10 strains from various broad-leafed trees in mountainous regions of Japan and Alaska (23). These strains had a red to orange color and could ferment glucose and produce amyloid compounds. Phaff et al. (23) referred to these strains as *Rhodozyma montanae*, but since a Latin diagnosis was not provided, the name was not valid. Later, this basidiomycetous yeast was described as *Phaffia rhodozyma* (20), and 67 additional strains were isolated from spring sap flows of *Betula verrucosa* in Russia (10).

The main carotenoid pigment produced by *P. rhodozyma* is astaxanthin, a compound that has not been found in other yeast species (3). Astaxanthin is economically important as an aquaculture feed component. This compound enhances pigmentation of fish and crustaceans (19) and is the most expensive feed ingredient in the aquaculture industry (12). Studies of the feasibility of *P. rhodozyma* as a dietary source of this pigment began almost 30 years ago (13). Such studies involved the development of methods for optimizing pigment production (24, 28), selection of hyperpigmented mutants (2), the use of chemical stimulants (11) or extracts of the filamentous fungus *Epicoccum nigrum* (5) in the growth medium, and genetic and metabolic engineering (21).

The sexual stage of *P. rhodozyma* was discovered in 1995, and its development is triggered when polyols are used as sole carbon sources (9). The teleomorphic state is characterized by the formation of a slender holobasidium with terminal basidiospores and was described as *Xanthophyllomyces dendrorhous*, with VKM Y-2786 (CBS 7918) as the type strain (9). Therefore, the name *X. dendrorhous* was used for the sexual form and *P. rhodozyma* was designated the asexual phase. The lack of sexual reproduction in the type strain of *P. rhodozyma* (CBS 5905) and differences in intergenic spacer (IGS) and internal transcribed spacer (ITS) sequences between CBS 5905 and VKM Y-2786 led Fell and Blatt (6) to propose that *P. rhodozyma*, represented solely by CBS 5905, was not conspecific with *X. dendrorhous*. The debate over this issue is not settled, and in another study isozyme analyses and restriction fragment length polymorphism and random amplified polymorphic DNA patterns suggested that CBS 5905 and five other strains of *X. dendrorhous* belong to a single species (27). The hypothesis that the anamorph *P. rhodozyma* is distinct, at the species level, from teleomorph *X. dendrorhous* also was favored by Kucsera et al. (14, 15), who based their arguments on the extensive chromosome length polymorphism seen in electrophoretic karyotypes (1, 22), the ability to produce respiration-

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TABLE 1. List of strains used in this study

Species	Strain	Other collection(s)	Origin
P. rhodozyma	CBS 5905^T	UCD 67-210, NRRL Y-10921	Exudate of *Fagus crenata*, Uehimi-yama, Kyoto, Japan
	PYCC 4172^T	UCD 67-210, NRRL Y-10921	
X. dendrorhous	CBS 9090	UCD 67-210.2	Culture derived from UCD 67-210
	CBS 7918^T	VKM Y-2786	Exudate of *Betula verrucosa*, Moscow, Russia
	CBS 5908	UCD 67-383	Exudate of *Alnus japonica*, Shinkai, Kiso, Japan
	CBS 6938	UCD 77-61	Sap of *Betula* sp. stumps, Finland
	CBS 7919	ATCC 24230, UCD 67-385	Exudate of *Betula tauschii*, Shinkai, Kiso, Japan
	ATCC 24228	UCD 68-653.3C	Exudate of *Betula papyrifera*, Rainbow Lake, Kenai Peninsula, AK
	ATCC 24229	UCD 67-202	Exudate of *Cornus brachypoda*, Kario-yama, Hiroshima, Japan
	ATCC 24201	UCD 67-203	Exudate of *Cornus brachypoda*, Kario-yama, Hiroshima, Japan
	ATCC 24261	UCD 67-484	Exudate of *Betula maximowicziana* at Yatani, Yamagata, Japan
	VKM Y-1652	KBP 2604	*Betula* sp., Novgorod region, Russia
	VKM Y-1654	KBP 2607	*Betula* sp., Komi Republic, Russia
	CRUB 0853[a]		Stroma of *Cyttaria hariotii* on *Nothofagus dombeyi*, Gutiérrez Lake, Patagonia, Argentina
	CRUB 1149[b]		Water from Lake Ilon, Patagonia, Argentina

[a] Same origin as strains CRUB 0924, CRUB 0919, and CRUB 1490 to CRUB 1499.
[b] Same origin as strain CRUB 1151.

deficient petite mutants spontaneously or following brief exposure to ethidium bromide, and a reexamination of the sexual form.

P. rhodozyma and *X. dendrorhous* have been found so far in tree exudates in the Northern Hemisphere at high altitudes and latitudes. No new strains of *P. rhodozyma*/*X. dendrorhous* have been isolated recently, and most of the current research is based on isolates first cultured in the 1960s and 1970s. This study is the first to document the occurrence of *Phaffia* yeasts in the Southern Hemisphere, and its main objective is to provide an adequate molecular and ecological characterization of the new isolates. Our working hypothesis is that *Phaffia* has a broader ecological distribution than the one presently known and that its occurrence in the Southern Hemisphere is linked to similar ecosystems in the Northern Hemisphere where *Phaffia* is known to occur. A deeper understanding of *Phaffia* biogeography, ecology, and molecular phylogeny is essential for the comprehension of many aspects of its biology and will facilitate the study of astaxanthin production within an evolutionary and ecological framework.

MATERIALS AND METHODS

Yeast isolation and reference cultures. Water samples from Lake Ilon (71°56′S, 41°11′W), Nahuel Huapi National Park (Patagonia, Argentina), were collected during January 2003 in aseptic flasks and filtered in situ through a membrane with 0.45-μm pores with a sterile Nalgene (Rochester, NY) filtering apparatus. The filters were placed in sterile petri dishes and kept at 4°C for approximately 36 h, until arrival at the laboratory, where they were placed on MYP agar (components, g/liter: malt extract, 7; yeast extract, 0.5; peptone-soytone, 2.5; agar, 15 [pH 5]) plus 200 mg/liter chloramphenicol and incubated at 15 to 17°C until colonies emerged.

Stromata of *Cyttaria hariotii*, an ascomycetous fungal parasite of *Nothofagus dombeyi* ("Coihue"), were collected aseptically in November to December (spring season) of 2003 on the shore of Gutiérrez Lake (71°43′S, 41°18′W). Fifteen *Cyttaria* fruiting bodies were collected and stored individually in sterile flasks to avoid cross contamination or crushing. Stromata were divided into three groups based on their maturation status (immature, early in maturation, and mature) and checked for yeasts. Stromata were weighed (5 to 50 g), checked for developmental stage, cut into small pieces (~2 × 2 cm), and placed in sterile plastic bags with a known volume of distilled sterile water in a water-to-fruiting-body ratio of 1 g/ml. *Cyttaria* fruiting bodies were manually crushed inside the plastic bags, which were then transferred to Erlenmeyer flasks and agitated at 20°C for 30 min at 300 rpm. The extract was diluted (10^{-3} or 10^{-4}), and aliquots (100 to 200 μl) were spread on MYP agar plates. These plates were incubated at 15 to 17°C for 5 to 7 days, and every pigmented colony was subcultured on MYP agar for purification. The production of amyloid compounds and the ability to ferment glucose were evaluated by using the procedures of Yarrow (30). The two strains isolated from water samples plus 13 selected strains recovered from different *Cyttaria* fruiting bodies were used for further studies. The ability to form the sexual stage was investigated by using previously described procedures (14). All of the pigmented isolates were maintained on potato dextrose agar at 4°C. In addition, strains identified as *X. dendrorhous* were deep frozen in liquid nitrogen.

In addition to these newly collected cultures, reference cultures were obtained from the Centraalbureau voor Schimmelcultures (CBS); the Department of Soil Biology, Moscow State University (KBP); the Portuguese Yeast Culture Collection (PYCC); and the University of California at Davis (UCD) (Table 1).

Molecular characterization. We used the microsatellite primed-PCR technique (MSP-PCR) for PCR fingerprinting. DNA extraction, PCR and electrophoresis conditions, and gel image analysis procedures were those of Libkind et al. (17). The primer employed was the synthetic oligonucleotide $(GTG)_5$. Measurements of the extent of DNA-DNA reassociation were made with a Gilford Response UV-VIS spectrophotometer (Ciba Corning Diagnostics Corp., Oberlin, OH) as described by Sampaio et al. (25). For sequence analysis, ribosomal DNA (rDNA) was amplified using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and LR6 (5′-CGCCAGTTCTGCTTACC-3′). Cycle sequencing of the 600- to 650-bp region of the 26S rDNA D1/D2 domain was done with forward primer NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′). The ITS region was sequenced using the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The IGS region was amplified using the forward primer LR11 (5′-TTACCACAGGGATAACTGGC-3′) and the reverse primer 5SR (5′-GGATCGGACGGGGCAGGGTGC-3′). For cycle sequencing the primers were LR12 (5′-CTGAACGCCTCTAAGTCAGAA-3′) (forward) and 5SR (reverse). Sequences were obtained with an Amersham Pharmacia (GE Healthcare, Buckinghamshire, England) ALF Express II automated sequencer by using standard protocols. Alignments were made with MegAlign (DNAstar Inc., Madison, WI) and visually corrected. Sequences available in GenBank were used for comparative purposes. The ITS sequences of the tree hosts of *P. rhodozyma*/*X. dendrorhous* also were obtained from GenBank. Phylogenetic analyses were made by using a heuristic maximum parsimony analysis (100 rounds of heuristic search with TBR branch swapping, starting from trees obtained by random addition of sequences with the MulTrees option on and the steepest descent option off). The molecular phylogenies were validated with 1,000 rounds of bootstrap analysis (7). Maximum parsimony analysis and bootstrap calculations were performed with PAUP* software (26).

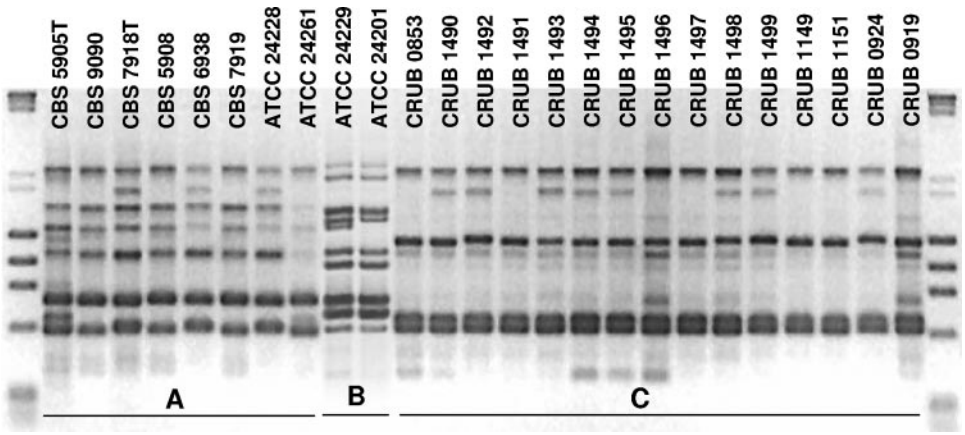

FIG. 1. MSP-PCR fingerprints of *X. dendrorhous* strains generated with microsatellite primer $(GTG)_5$. A, B, and C indicate the three main fingerprints. The first and last lanes correspond to the molecular size marker (λ DNA cleaved with HindIII and ΦX174 DNA cleaved with HaeIII).

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are DQ661021 to DQ661034.

RESULTS

Patagonian isolates. We isolated two orange-pigmented strains (CRUB 1149 and CRUB 1151) that formed amyloid compounds and gave positive results in the glucose fermentation test from high-altitude oligotrophic lakes of the Nahuel Huapi National Park (Patagonia). Based on sequences of the D1/D2 domains of large-subunit rDNA both strains were closely related to *P. rhodozyma*/*X. dendrorhous*, i.e., there were no sequence mismatches in the comparison with the type strain of *Phaffia rhodozyma.* The *Phaffia*/*Xanthophyllomyces* isolates from the Northern Hemisphere were found in slime fluxes of trees or tree stumps remaining after logging, so we hypothesized that the aquatic environment from which these two isolates were obtained was not their original habitat. Assuming that the yeast is primarily associated with ecological niches where fermentable sugars are present, we surveyed the plant biota surrounding the high-altitude lakes of the Nahuel Huapi National Park for the occurrence of slime fluxes. The main trees occurring in this region are *Nothofagus dombeyi* and *Nothofagus pumilio*, but trees of neither of these species had signs of active or dry slime fluxes.

While we were surveying the trees for slime fluxes, our attention was drawn to *Cyttaria hariotii*, an ascomycetous fungal parasite of *Nothofagus* spp., whose mycelium develops inside the trunk and the branches of the tree. As a reaction to the infection, persistent tumors are formed. Fructifications (stromata) are produced annually on the tumors. The stromata of *C. hariotii* are globose and yellow to orange, measure 2 to 6 cm in diameter, and mature in November-December (8). Yeasts were present at all maturation stages of the *Cyttaria* stromata. Mature fruiting bodies yielded the highest yeast counts (above 9×10^4 CFU/g [wet weight]), while immature stromata and those in the first stages of maturation had 900 and 2,000 CFU/g, respectively. The proportion of pigmented yeasts in *C. hariotii* gradually declined with maturation. In late maturation stages, most of the yeast isolates were fermentative ascomycetes of the genera *Candida* and *Saccharomyces* and only 1 to 5% of the colonies on the isolation plates were pigmented (red to orange cultures). Overall, we obtained 40 pigmented isolates, of which 36 produced amyloid compounds. Glucose was fermented by 33 of the strains that formed amyloid compounds.

Based on MSP-PCR, the Patagonian isolates belonged to a single species, as the profiles of the various strains were basically identical (Fig. 1). The sequence of the D1/D2 domains of large-subunit rDNA of CRUB 0853 (DQ661033) also was identical to that of the aquatic strain CRUB 1149 and that of the type strain of *Phaffia rhodozyma*.

Selected Patagonian (CRUB) isolates and the type strains of *P. rhodozyma* and *X. dendrorhous* were inoculated on ribitol agar and examined for the ability to develop basidia and basidiospores. All 15 South American isolates and the type strain of *X. dendrorhous* produced the sexual form, but the type strain of *P. rhodozyma* did not (14). Therefore, the Patagonian isolates were identified as *X. dendrorhous* rather than as *P. rhodozyma*.

The proportion of *X. dendrorhous* strains recovered depended on the maturity of the stroma. Mature stromata yielded the most isolates (27 strains), none were recovered from immature stromata, and six were isolated from stromata in the first stages of maturation. *N. dombeyi*, the host tree of *C. hariotii*, was surveyed for *Phaffia*/*Xanthophyllomyces*, but no cultures were obtained from leaves, buds, or bark. Additional water samples from nearby lakes also were negative.

Molecular phylogeny and host specificity. In spite of their overall similarity, the fingerprints obtained with primer $(GTG)_5$ (Fig. 1) could be used to subdivide the isolates into three groups. The first group (A) contained the type strains of *P. rhodozyma* and *X. dendrorhous* and several other strains from different geographic areas. The second group (B) included two strains of *X. dendrorhous* isolated by Phaff et al. (23), and the third group (C) included the Patagonian isolates. The same set of strains also was evaluated with primer M13 (data not shown), and the same three groups were identified.

We used ITS sequence data and a maximum parsimony analysis to evaluate the relatedness of the *Phaffia*/*Xanthophyllomyces* isolates (Fig. 2A). We expanded the ITS data set of Fell and Blatt (6) from 6 to 18 sequences. Four of the new sequences are from Patagonian isolates, and the remaining

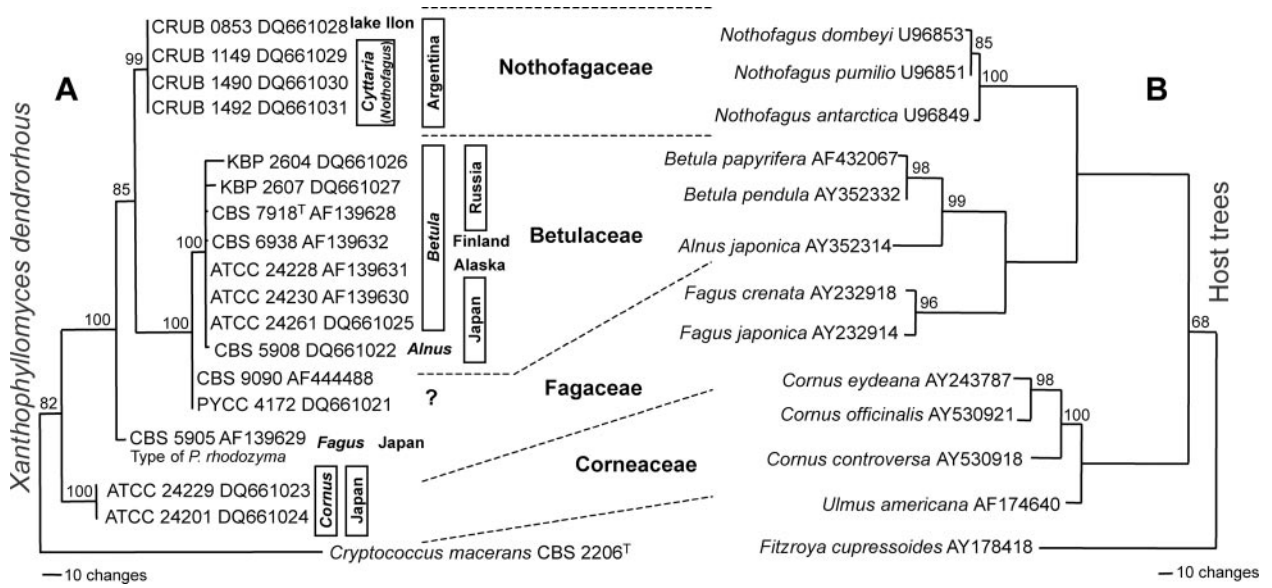

FIG. 2. Phylogenetic relationships between different isolates of *X. dendrorhous* and their plant hosts. The consensus trees were based on alignments of the complete ITS region (ITS 1, 5.8S rDNA, and ITS 2) and were generated via maximum parsimony analysis in PAUP*. The topology of the *X. dendrorhous* tree was rooted with *Cryptococcus macerans*, and that of the plant host tree was rooted with *Fitzroya cupressoides*. Names of plant families, localities, and host trees are indicated in the center of the figure. Numbers on the branches are bootstrap values (1,000 replicates; values below 50% are not shown). GenBank accession numbers of the sequences are indicated after strain numbers.

eight sequences are from Russian (two sequences) and Japanese (six sequences) strains isolated by W. I. Golubev and H. J. Phaff, respectively. The type strain of *X. dendrorhous*, which was isolated from *Betula verrucosa* in Russia, clustered with all of the other strains of this species originating from Betulaceae (*Alnus* and *Betula*) but not with the strains isolated from *Cornus* (Cornaceae) and *Nothofagus* (Nothofagaceae), the plant host of *Cyttaria* (Fig. 2A). The isolated position occupied by the type strain of *P. rhodozyma* CBS 5905 in the phylogenetic tree could be due to its association with a different tree (*Fagus crenata*, Fagaceae). Strains PYCC 4172 and CBS 9090 clustered together at the base of the clade composed of isolates from Betulaceae (Fig. 2A).

The original strain number of the *P. rhodozyma* type strain was UCD 67-210. This strain was sent in 1968 to the CBS culture collection (CBS 5905) (V. Robert, personal communication) and in 1976 to the NRRL culture collection (NRRL Y-10921) (C. Kurtzman, personal communication). From this last culture collection it was sent to the Portuguese Yeast Culture Collection (PYCC 4172) in 1982. Therefore, CBS 5905 and PYCC 4172 should have identical ITS sequences as they are subcultures of the same strain. The original number for CBS 9090 was UCD 67-210.2. According to the curator of the Phaff Yeast Culture Collection, University of California—Davis, subcultures of UCD 67-210 were lyophilized on several different occasions between 1968 and 1986 and UCD 67-210.2 was one of these stocks (K. Boundy-Mills, personal communication).

Given the apparent association of various ITS phylotypes of *X. dendrorhous* with different tree families, we tested the hypothesis that different populations of *X. dendrorhous* colonize different trees and analyzed the evolutionary relationships of the genera of trees that are known to harbor *Xanthophyllomyces* (Fig. 2B). We used complete ITS sequences, and in general, good agreement was observed between the fungal and the plant phylogenetic trees (Fig. 2).

Our ITS analysis was complemented with a phylogenetic analysis of the rDNA IGS region, a region previously evaluated by Fell and Blatt (6). They found that for *X. dendrorhous*, differences in sequences and the occurrence of gaps could be used to differentiate strains and for population-level studies. Our IGS alignment of 657 nucleotides differed substantially from the one presented by Fell and Blatt (6), with numerous short nucleotide repeats and gaps contributing to distinct alignment options. Similar difficulties also were reported by Fell and Blatt (6) for their IGS alignment. In spite of this we found, in our analysis, that the sequence of the Patagonian isolate was phylogenetically closer to that of CBS 5905 than to any of the already known sequences (Fig. 3).

DISCUSSION

New habitat. In this report we describe, for the first time, the isolation of *X. dendrorhous* in the Southern Hemisphere and we identify a new habitat for this yeast, the stromata of *C. hariotii*. The association of *X. dendrorhous* with stromata of *C. hariotii* was unexpected since all other reports of the occurrence of this yeast concerned tree exudates. The genus *Cyttaria* is associated exclusively with *Nothofagus* spp., and its 10 species occur solely in South America (Argentina and Chile) and Australasia (Australia and New Zealand) (8). It would be interesting to determine if *X. dendrorhous* is present on other species of *Cyttaria*, especially the Australasian taxa.

A peculiar characteristic of *X. dendrorhous* is its ability to ferment simple sugars. When mature, the fruiting bodies of *C. hariotii* have a sugar content of almost 10% (mainly D-glucose,

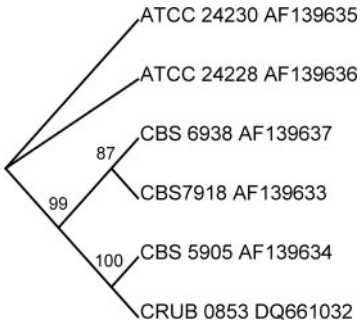

FIG. 3. Unrooted tree depicting the phylogenetic relationships between different isolates of *X. dendrorhous* based on IGS sequences and maximum parsimony analysis performed in PAUP*. Numbers on the branches are bootstrap values (1,000 replicates; values of <50% are not shown). GenBank accession numbers of the sequences are indicated after strain numbers.

fructose, and sucrose) and contain polyols such as glycerol, D-mannitol, and D-arabinitol (16). Although we recovered two isolates from freshwater in a high-altitude lake, surveys of pigmented yeasts in 15 other Patagonian aquatic environments did not yield any additional isolates of this species (17). We hypothesize that the primary habitat of the two aquatic isolates also was *C. hariotii* because *Nothofagus* spp. infected with the fungus surround the lake from which the two strains were obtained. Mature stromata normally fall to the ground where they decompose, but stromata falling from trees on the lake shore may fall in the water, where they float for some time.

South American populations. MSP-PCR fingerprinting has been used to type fungal strains by several research groups. In most cases species-specific PCR patterns are obtained (17, 25, 29), but in some instances this approach results in intraspecific differentiation (4, 18). Our results suggest that primers $(GTG)_5$ and M13 are useful to discriminate populations of *Phaffia*/*Xanthophyllomyces*. The South American isolates formed a single, homogeneous cluster (group C) that did not include strains from other geographic areas. Thus, the Patagonian strains seem to represent a genetically uniform population even though they were isolated from different locations. Most of the studied strains from the Northern Hemisphere belonged to group A, which includes strains from Japan, Finland, and Russia. However, Japanese isolates also are found in group B, so the intraspecific polymorphism detected by MSP-PCR may not be strictly correlated with the geographic origin of the isolate.

Host specificity model. The comparative analysis of ITS sequence data from *Xanthophyllomyces*/*Phaffia* and their tree hosts suggests that different yeast lineages colonize different trees. Specific (insect?) vectors colonize different trees and could contribute to the observed separation. Since the two phylogenetic trees (Fig. 2) have similar topologies, we hypothesize that the observed phylogenetic pattern of *X. dendrorhous* follows the speciation of its plant hosts. However, two unanswered questions arise from these findings: (i) are the isolation mechanisms stronger or weaker than those that contribute to the unity of *X. dendrorhous* as a species, and (ii) if the forces contributing to the isolation of the several *Xanthophyllomyces* lineages are dominant, is speciation currently in progress? Further studies are needed to elucidate the degree of gene flow between representatives of the different *Xanthophyllomyces* lineages.

From the evolutionary point of view one can postulate that the present association of *Xanthophyllomyces* with *Cyttaria* derived from an earlier association of the yeast with *Nothofagus*. The sister relationship between Nothofagaceae and Betulaceae plus Fagaceae (Fig. 2B) was also observed in studies of angiosperm phylogeny (Angiosperm Phylogeny Website, version 7, May 2006, P. F. Stevens [http://www.mobot.org/MOBOT/research/APweb]) and corresponds very well with the phylogeny of *X. dendrorhous* strains originating from these three plant families (Fig. 2A). The two most divergent and basal strains of *X. dendrorhous* are those originating from *Cornus* (Fig. 2A), a divergent and ancestral genus in the phylogenetic analysis of the host trees (Fig. 2B).

In spite of the considerable number of strains that were analyzed, the type strain of *P. rhodozyma* remains phylogenetically isolated. According to our model this result is not connected with the number of strains employed in the analysis but is rather a direct consequence of the habitat (tree species) from which each isolate was recovered. We anticipate that the isolated placement of the type strain of *P. rhodozyma* will change only if new isolates from *Fagus* are obtained. Thus, the isolated position of the type strain of *P. rhodozyma* does not necessarily mean that CBS 5905 is not conspecific with (and the asexual form of) *X. dendrorhous*.

Interpretations of previous studies with IGS sequence data suggested that this approach was adequate to reflect the biogeography of *X. dendrorhous* since ATCC 24228 and ATCC 24230, the two Pacific Rim strains, were separated from CBS 6938 and CBS 7918, the two European strains (6). Moreover, the phylogenetic differences were regarded as indications that *X. dendrorhous* includes two varieties, and the isolated position of CBS 5905 was interpreted as additional evidence that *P. rhodozyma* and *X. dendrorhous* are not conspecific. Our IGS analysis partially supports the conclusions of Fell and Blatt (6). We think that the IGS data are promising for a biogeographic study of isolates from Betulaceae since the Pacific Rim strains are distinct from the European isolates. However the association between the Patagonian isolate and CBS 5905 challenges the two-species concept. We anticipate that IGS sequence typing might be a useful approach for the characterization of isolates within a given host group.

Was the type strain of *P. rhodozyma* mislabeled? The relationship between CBS 5905, PYCC 4172, and CBS 9090 remains unresolved. According to the records from several culture collections, all of these strains are derived from UCD 67-210, the original type strain of *P. rhodozyma*, but the expected identity of these three strains is not seen with the molecular characters. The hypothesis that one or more of these strains has been mislabeled cannot yet be discarded, although we could not identify the strain that might represent the source of such mislabeling (in terms of either identical ITS sequences or, for CBS 5905, the absence of the sexual stage). Golubev (9) reported that VKM Y-2274 (type strain of *P. rhodozyma*) could form basidia and basidiospores. The VKM strain was obtained from the UCD collection in 1975 (W. I. Golubev, personal communication). However, Kucsera et al. (14) reported that CBS 5905 could not form a sexual stage. We confirmed that

CBS 5905 is asexual and that PYCC 4172 and CBS 9090 are sexually fertile. Another possible explanation is that prolonged maintenance in culture collections resulted in a divergence of CBS 5905 from the other two strains. The oldest copy of the original culture is CBS 5905, which dates from 1968 and is the most divergent, while the VKM culture was obtained in 1975 and the PYCC culture in 1976 (via the NRRL culture collection). Such an extensive divergence (including loss of the ability to form sexual structures) has not previously been documented for yeasts. Further studies with additional molecular markers and the determination if there are multiple copies of the ITS region within each strain are needed to adequately clarify this situation.

***P. rhodozyma* or *X. dendrorhous*?** Our results are not consistent with the hypothesis that *P. rhodozyma* and *X. dendrorhous* represent different species (6, 15). Most of the differences in the sequence data can be attributed to intraspecific variation associated with host specificity. The issue of the relatedness of the type strains of *P. rhodozyma* (CBS 5905) and *X. dendrorhous* (CBS 7918) among themselves and with Patagonian isolate CRUB 0853 also was evaluated with genomic DNA-DNA reassociation experiments. These reassociation values all were high, which is consistent with the hypothesis that only a single species is present. For CBS 5905 × CBS 7918, DNA-DNA reassociation values ranged from 96 to 100%, values for CBS 5905 × CRUB 0853 ranged from 79 to 100%, and values for CBS 7918 × CRUB 0853 ranged from 79 to 90%.

In conclusion, we think that nomenclatural changes are premature and support the position that *P. rhodozyma* is the asexual stage of *X. dendrorhous.* We think that a better understanding of the processes underlying the observed differences within *X. dendrorhous* might have relevance since they could shed new light on the evolution of the astaxanthin biosynthetic pathway and on the ecological conditions that selected for this trait. Finally, we do not reject the hypothesis that speciation is in progress in *X. dendrorhous*. The driving force underlying such a process could be isolation due to host specificity of the different lineages of *X. dendrorhous.* We anticipate that multilocus sequence typing might be useful in assessing the degree of genetic isolation of the various lineages of *X. dendrorhous* identified in this report.

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REFERENCES

1. **Adrio, J. L., M. Lopez, J. Casqueiro, C. Fernández, and M. Veiga.** 1995. Electrophoretic karyotype of the astaxanthin-producing yeast *Phaffia rhodozyma*. Curr. Genet. **27:**447–450.
2. **An, G. H., D. B. Schuman, and E. A. Johnson.** 1989. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl. Environ. Microbiol. **55:**116–124.
3. **Andrewes, A. G., H. J. Phaff, and M. P. Starr.** 1976. Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry **15:**1003–1007.
4. **Caruso, M., A. Capece, G. Salzano, and P. Romano.** 2002. Typing of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains from Aglianico wine. Lett. Appl. Microbiol. **34:**323–328.
5. **Echavarri-Erasun, C., and E. A. Johnson.** 2004. Stimulation of astaxanthin formation in the yeast *Xanthophyllomyces dendrorhous* by the fungus *Epicoccum nigrum*. FEMS Yeast Res. **4:**511–519.
6. **Fell, J. W., and G. M. Blatt.** 1999. Separation of strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis. J. Ind. Microbiol. Biotechnol. **23:**677–681.
7. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
8. **Gamundí, I. J., and R. M. Lederkremer.** 1989. Los hongos andino-patagónicos del género *Cyttaria*. Sus hidratos de carbono. Cienc. Investig. **43:**4–13.
9. **Golubev, W. I.** 1995. Perfect state of *Rhodomyces dendrorhous* (*Phaffia rhodozyma*). Yeast **11:**101–110.
10. **Golubev, W. I., I. P. Bab'eva, V. M. Blagodatskaya, and I. S. Reshetova.** 1977. Taxonomic study of yeasts isolated from spring sap flows of birch (*Betula verrucosa* Ehrh.). Mikrobiologia **46:**564–568.
11. **Gu, W. L., G. H. Hwan, and E. A. Johnson.** 1997. Ethanol increases carotenoid production in *Phaffia rhodozyma*. J. Ind. Microbiol. Biotechnol. **19:**114–117.
12. **Johnson, E. A., and W. A. Schroeder.** 1995. Microbial carotenoids, p. 297–326. *In* A. Fiechter (ed.), Advances in biochemical engineering/biotechnology. Springer, Heidelberg, Germany.
13. **Johnson, E. A., D. E. Conklin, and M. J. Lewis.** 1977. The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonids and crustaceans. J. Fish. Res. Board Can. **34:**2417–2421.
14. **Kucsera, J., I. Pfeiffer, and I. Ferenczy.** 1998. Homothallic life cycle in the diploid red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Antonie Leeuwenhoek **73:**163–168.
15. **Kucsera, J., I. Pfeiffer, and K. Takeo.** 2000. Biology of the red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Mycoscience **41:**195–199.
16. **Lederkremer, R. M., and M. E. Ranalli.** 1967. Hidratos de carbono en hongos de la Patagonia Argentina. Azúcares simples y polioles en *Cyttaria hariotii* Fischer. An. Asoc. Quím. Arg. **55:**199–203.
17. **Libkind, D., S. Brizzio, A. Ruffini, M. Gadanho, M. R. van Broock, and J. P. Sampaio.** 2003. Molecular characterization of carotenogenic yeasts from aquatic environments in Patagonia, Argentina. Antonie Leeuwenhoek **84:**313–322.
18. **Meyer, W., T. G. Mitchell, E. Z. Freedman, and R. Vilgalys.** 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J. Clin. Microbiol. **31:**2274–2280.
19. **Meyers, S. P.** 1994. Developments in world aquaculture, formulations, and the role of carotenoids. Pure Appl. Chem. **66:**1069–1076.
20. **Miller, M. W., M. Yoneyama, and M. Soneda.** 1976. *Phaffia*, a new yeast genus in the Deuteromycotina (Blastomycetes). Int. J. Syst. Bacteriol. **26:**286–291.
21. **Misawa, N., and H. Shimada.** 1997. Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J. Biotechnol. **59:**169–181.
22. **Nagy, Á., N. Garamszegi, C. Vágvölgyi, and L. Ferenczy.** 1994. Electrophoretic karyotypes of *Phaffia rhodozyma* strains. FEMS Microbiol. Lett. **123:**315–318.
23. **Phaff, H. J., M. W. Miller, M. Yoneyama, and M. Soneda.** 1972. A comparative study of the yeast florae associated with trees on the Japanese islands and on the west coast of North America, p. 759–774. *In* G. Terui (ed.), Proceedings of the 4th IFS: Fermentation Technology Today Meeting. Society of Fermentation Technology, Osaka, Japan.
24. **Ramirez, J., H. Gutierrez, and A. Gschaedler.** 2001. Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology. J. Biotechnol. **88:**259–268.
25. **Sampaio, J. P., M. Gadanho, S. Santos, F. Duarte, C. Pais, A. Fonseca, and J. W. Fell.** 2001. Polyphasic taxonomy of the genus *Rhodosporidium*: *R. kratochvilovae* and related anamorphic species. Int. J. Syst. Evol. Microbiol. **51:**687–697.
26. **Swofford, D. L.** 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland, MA.
27. **Varga, J., C. Vágvölgyi, A. Nagy, L. Pfeiffer, and L. Ferenczy.** 1995. Isoenzyme, restriction fragment length polymorphism, and random amplified polymorphic DNA characterization of *Phaffia rhodozyma* Miller et al. Int. J. Syst. Bacteriol. **45:**173–177.
28. **Vazquez, M., and A. M. Martin.** 1998. Optimization of *Phaffia rhodozyma* continuous culture through response surface methodology. Biotechnol. Bioeng. **57:**314–320.
29. **Wuczkowski, M., and H. Prillinger.** 2004. Molecular identification of yeasts from soils of the alluvial forest national park along the river Danube downstream of Vienna, Austria ("National park Donauauen"). Microbiol. Res. **159:**263–275.
30. **Yarrow, D.** 1998. Methods for the isolation, maintenance and identification of yeasts, p. 77–100. *In* C. P. Kurtzman and J. W. Fell (ed.), The yeasts: a taxonomic study, 4th ed. Elsevier, Amsterdam, The Netherlands.